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(54) Polypeptides, process for their microbial production, intermediates therefor and compositions containing them.

(57) Microbially produced mature human fibroplast Interferon and means used in its production, i.e. recombinant DNA molecules coding for the amino acid sequences of fibroplast Interferon, vectors capable of expressing fibroblast Interferon in microbial host organisms and host organisms transformed with these vectors.
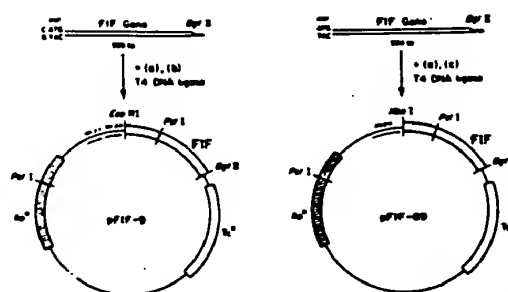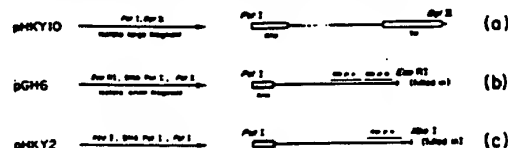
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ACTORUM AG

Polypeptides, process for their microbial production,
intermediates therefor and compositions containing them

5

10   The present invention relates to the field of re-
combinant DNA technology, i.e. to processes used in recom-
binant DNA technology and to products obtained by these
processes.

15   In a more detailed aspect the present invention re-
lates to polypeptides, specifically to mature human fibro-
blast interferon, to pharmaceutical compositions containing
them and to a process for their preparation which comprises
causing a culture of a microorganism transformed with a
20 replicable microbial expression vehicle capable of expres-
sing said polypeptides to grow up and express said poly-
peptides. The present invention also comprises the expres-
sion vehicles used in this process and the novel micro-
organisms containing these expression vehicles as well as
25 the processes for their preparation. Finally, the invention
relates to DNA sequences comprising sequences coding for
the amino acid sequence of a mature human fibroblast inter-
feron.

30   Background of the invention

        Human fibroblast interferon (FIF) is a protein which
exhibits antiviral as well as a wide range of other bio-
logical activities (for review see W.E. Stewart II, The
35 Interferon System, Springer-Verlag, New York-Wien, 1979).

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It has reportedly been purified to homogeneity as a
single polypeptide with a molecular weight of 19000 - 20000
having a specific activity of $2-10 \times 10^8$ units/mg (E.
Knight, Proc. Natl. Acad. Sci. USA 73, 520-523 [1976];

5 W. Berthold et al., J. Biol. Chem. 253, 5206-5212 [1978]).
The sequence of the 13 NH_2-terminal amino acids of FIF
has been determined to be Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-
Leu-Gln-Arg-Ser-Ser- (E.Knight et al., Science 207, 525-
526 [1980]). Houghton et al. (Nucleic Acids Res. 8, 1913-

10 1931 [1980]) have used synthetic deoxyoligonucleotides
(predicted from this amino acid sequence) to determine the
sequence of the 276 5'-terminal nucleotides of FIF mRNA.
Taniguchi et al. (Nature 285, 547-549 [1980]; Gene 10,
11-15 [1980]) and Derynck et al. (Nature 285, 542-547

15 [1980]) have recently been able to identify the nucleo-
tide sequence of cloned cDNA copies of FIF mRNA in E. coli
and have deduced therefrom the complete amino acid sequence
of human FIF including a 21 amino acids signal sequence.
The mature peptide is 166 amino acids long. Finally,

20 Taniguchi et al. (Proc. Natl. Acad. Sci. USA 77, 5230-5233
[1980]) have constructed a plasmid that directs expression
in E. coli of the human FIF gene yielding mature FIF.

With the advent of recombinant DNA technology, the
25 controlled microbial production of an enormous variety of
useful polypeptides has become possible. Already in hand
are bacteria modified by this technology to permit the
production of such polypeptide products such as somatosta-
tin, the A and B chains of human insulin, human growth

30 hormone (Itakura et al., Science 198, 1056-1063 [1977];
Goeddel et al., Nature 281, 544-548 [1979]). More recently,
recombinant DNA techniques have been used to occasion the
bacterial production of proinsulin, thymosin α_1 and
leukocyte interferon.

35

The workhorse of recombinant DNA technology is the
plasmid, a non-chromosomal loop of double-stranded DNA
found in bacteria and other microbes, oftentimes in multiple

copies per cell. Included in the information encoded in
the plasmid DNA is that required to reproduce the plasmid
in daughter cells (i.e., a "replicon") and ordinarily, one
or more selection characteristics such as, in the case of
5 bacteria, resistance to antibiotics which permit clones of
the host cell containing the plasmid of interest to be
recognized and preferentially grown in selective media.
The utility of plasmids lies in the fact that they can be
specifically cleaved by one or another restriction endo-
10 nuclease or "restriction enzyme", each of which recognizes
a different site on the plasmidic DNA. Thereafter hetero-
logous genes or gene fragments may be inserted into the
plasmid by endwise joining at the cleavage site or at
reconstructed ends adjacent to the cleavage site. DNA
15 recombination is performed outside the cell, but the resul-
ting "recombinant" plasmid can be introduced into it by a
process known as transformation and large quantities of
the heterologous gene-containing recombinant plasmid are
obtained by growing the transformant. Moreover, where the
20 gene is properly inserted with reference to portions of
the plasmid which govern the transcription and transla-
tion of the encoded DNA message, the resulting expression
vehicle can be used to actually produce the polypeptide
sequence for which the inserted gene codes, a process
25 referred to as expression.

Expression is initiated in a region known as the pro-
moter which is recognized by and bound by RNA polymerase.
In some cases, as in the tryptophan or "trp" promoter pre-
30 ferred in the practice of the present invention, promoter
regions are overlapped by "operator" regions to form a
combined promoter-operator. Operators are DNA sequences
which are recognized by so-called repressor proteins which
serve to regulate the frequency of transcription initiation
35 at a particular promoter. The polymerase travels along the
DNA, transcribing the information contained in the coding
strand from its 5' to 3' end into messenger RNA which is in
turn translated into a polypeptide having the amino acid

sequence for which the DNA codes. Each amino acid is enco-
ded by a nucleotide triplet or "codon" within what may for
present purposes be referred to as the "structural gene",
i.e. that part which encodes the amino acid sequence of
5 the expressed product. After binding to the promoter, the
RNA polymerase first transcribes nucleotides encoding a
ribosome binding site, then a translation initiation or
"start" signal (ordinarily ATG, which in the resulting
messenger RNA becomes AUG), then the nucleotide codons
10 within the structural gene itself. So-called stop codons
are transcribed at the end of the structural gene where-
after the polymerase may form an additional sequence of
messenger RNA which, because of the presence of the stop
signal, will remain untranslated by the ribosomes. Ribo-
15 somes bind to the binding site provided on the messenger
RNA, in bacteria ordinarily as the mRNA is being formed,
and themselves produce the encoded polypeptide, beginning
at the translation start signal and ending at the previ-
ously mentioned stop signal. The desired product is pro-
20 duced if the sequences encoding the ribosome binding site
are positioned properly with respect to the AUG initiaton
codon and if all remaining codons follow the initiaton
codon in phase. The resulting product may be obtained by
lysing the host cell and recovering the product by appro-
25 priate purification from other bacterial protein.

While isolation from donor fibroblasts has provided
sufficient material for partial characterization and limi-
ted clinical studies with homogeneous leukocyte interferon,
30 it is a totally inadequate source for the amounts of inter-
feron needed for large scale clinical trials and for broad
scale prophylactic and/or therapeutic use thereafter.
Indeed, presently clinical investigations employing human
fibroblast-derived interferons in antitumor and antiviral
35 testing have principally been confined to crude (<1 per-
cent pure) preparations of the material, and long lead
times for the manufacture of sufficient quantities, even
at unrealistic price levels, have critically delayed

investigation on an expanded front.

We perceived that application of recombinant DNA
technology would be the most effective way of providing
5 large quantities of fibroblast interferon which, despite
the absence in material so produced of the glycosylation
characteristic of human-derived material, could be employed
clinically in the treatment of a wide range of viral and
neoplastic diseases and have succeeded in producing mature
10 human fibroblast interferon microbially, by constructing
a gene therefor which could then be inserted in microbial
expression vehicles and expressed under the control of
microbial gene regulatory controls.

15   Our approach to obtaining a fibroblast gene involved
the following tasks:

1. Partial amino acid sequences of human fibroblast
interferon were used to construct sets of synthetic DNA
20 probes the codons of which, in the aggregate, represented
all the possible combinations capable of encoding the
partial amino acid sequences.

2. Bacterial colony banks were prepared containing
25 complementary DNA (cDNA) from induced messenger RNA. The
probes of part (1) were used to prime the synthesis of
radio-labelled single stranded cDNA for use as hybridization
probes. The synthetic probes would hybridize with induced
mRNA as template and be extended by reverse transcription
30 to form induced, radio-labelled cDNA. Clones from the
colony bank that hybridized to radio-labelled cDNA obtained
in this manner have been investigated further to confirm
the presence of a full-length interferon encoding gene.
Any partial length putative gene fragment obtained was
35 itself used as a probe for the full-length gene.

3. The full-length gene obtained above was tailored, using synthetic DNA, to eliminate any leader sequence that might prevent microbial expression of the mature poly-peptide and to permit appropriate positioning in an expres-
5 sion vehicle relative to start signals and the ribosome binding site of a microbial promoter. Expressed interferon was purified to a point permitting confirmation of its character and determination of its activity.

10   In applying methods of recombinant DNA technology as outlined above a series of replicable plasmidic expression vehicles have been constructed which direct the high level synthesis in transformant microorganisms of a mature poly-peptide with the properties of authentic human fibroblast
15 interferon. The product polypeptide exhibits the amino acid sequence of such interferon and is active in in vitro testing despite the lack of glycosylation characteristic of the human-derived material. Reference herein to the "expression of mature fibroblast interferon" connotes the
20 bacterial or other microbial production of an interferon molecule containing no glycosyl groups or a presequence that immediately attends mRNA translation of the human fibroblast interferon genome. Mature fibroblast interferon, according to the present invention, is immediately
25 expressed from a translation start signal (ATG) which also encodes the first amino acid codon of the natural product. The presence or absence of the methionine first amino acid in the microbially expressed product is governed by a kinetic phenomenon dependent on fermentation growth condi-
30 tions and/or levels of expression in the transformant host. Mature fibroblast interferon could be expressed together with a conjugated protein other than the conventional leader, the conjugate being specifically cleavable in an intra- or extracellular environment (see British Patent
35 Publication No. 2007676A). Finally, the mature interferon could be produced in conjuction with a microbial "signal" peptide which transports the conjugate to the cell wall, where the signal is processed away and the mature poly-

peptide secreted.

Figures 1 to 5 appended hereto are described in the
detailed text infra. Figure 6 schematically depicts the
5 construction of plasmids coding for the direct expression
of mature fibroblast interferon. Restriction sites and
residues are as shown ("Pst I",etc.). "Ap^R" and "Tc^R"
connote portions of the plasmid which express, respecti-
vely, ampicillin and tetracycline resistance. The legend
10 "p o" is an abbreviation for "promoter operator".

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A.　　Microorganisms employed

15

The work described involved use of the microorganism
E. coli K-12 strain 294 (end A, thi$^-$, hsr$^-$, hsm$_k^+$), as
described in British Patent Publication No. 2055382 A. This
strain has been deposited with the American Type Culture
on Oct.28,1978
20 Collection, ATCC Accession No. 31446. All recombinant DNA
work was performed in complicance with applicable guidelines
of the National Institutes of Health.

The invention although described in its most preferred
25 embodiments with reference to E. coli K-12 strain 294,
defined above, comprises also other known E. coli strains
such as E. coli B, E. coli x 1776 and E. coli W 3110,
or other microbial strains many of which are deposited
and (potentially) available from recognized microorganism
30 depository institutions, such as the American Type Culture
Collection (ATCC). See also German Offenlegungsschrift
2644432. These other microorganisms include, for example,
Bacilli such as Bacillus subtilis and other enterobac-
teriaceae among which can be mentioned as examples Sal-
35 monella typhimurium and Serratia marcescens, utilizing
plasmids that can replicate and express heterologous gene
sequences therein. Yeast, such as Saccharomyces cerevisiae,
may also be employed to advantage as host organism in the

preparation of the interferon protein hereof by expression
of genes coding therefor under the control of a yeast
promoter.

5   B.   General methods

Restriction enzymes were purchased from New England
Biolabs and used as directed. Plasmid DNA was prepared by
a standard cleared lysate procedure (D.B. Clewell, J. Bac-
10 teriol. 110, 667-676 [1972]) and purified by column chroma-
tography on Biogel A-50M. DNA sequencing was performed
using the method of Maxam and Gilbert (Methods Enzymol.
65, 499-560 [1980]). DNA restriction fragments were iso-
lated from polyacrylamide gels by electroelution. DNA
15 fragments were radiolabeled for use as hydridization probes
by the random calf thymus DNA priming procedure of Taylor
et al. (Biochim. Biophys. Acta 442, 324-330 [1976]). In
situ colony hybridizations were performed by the Grunstein-
Hogness procedure (Proc. Natl. Acad. Sci. USA 72, 3961-
20 3965 [1975]).

C.   Chemical synthesis of deoxyoligonucleotides

The deoxyoligonucleotides were synthesized by the
25 modified phosphotriester method in solution (Crea et al.,
Proc. Natl. Acad. Sci. USA 75, 5765-5769 [1978]), using
trideoxynucleotides as building blocks (Hirose et al.,
Tetrahedron Letters 28, 2449-2452 [1978]). The materials
and general procedures were similar to those described
30 by Crea et al., Nucleic Acids-Res. 8, 2331-2348 [1980].
The six pools of primers (Figure 1) containing four
dodecanucleotides each were obtained by separately coupling
two hexamer pools (of two different 5'-terminal sequences
each) with three different hexamer pools (of two different
35 3'-terminal sequences each).

D. Induction of fibroblasts

Human fibroblasts (cell line GM-2504A) were grown as described previously by Pestka et al., Proc. Natl. Acad.
5 Sci. USA 72, 3898-3901 [1975]. Growth medium (Eagle's minimal essential medium containing 10% fetal calf serum) was removed from roller bottles ·(850 cm^3) and replaced with 50 ml growth medium containing 50 µg/ml of poly(I): poly(C) and 10 µg/ml cycloheximide. This induction medium
10 was removed after 4 hours at 37°C and cell monolayers were washed with phosphate buffered saline (PBS; 0.14M NaCl, 3mM KCl, 1.5 mM KH_2PO_4, 8mM Na_2HPO_4). Each bottle was incubated at 37°C with 10 ml of a trypsin - EDTA solution (Gibco 610-5305) until cells were detached, and
15 fetal calf serum was added to a concentration of 10%. Cells were spun for 15 minutes at 500 x g and pellets were resuspended in PBS, pooled, and resedimented. Cells were frozen in liquid nitrogen. Approximately 0.17 g of cells were obtained per roller bottle.

20
E. Preparation and assay of interferon mRNA

Poly(A)-containing mRNA was prepared from human fibro-blasts by phenol extractions and oligo(dT)-cellulose
25 chromatography as described by Green et al. (Arch. Bio-chem. Biophys. 172, 74-89 [1975]). The poly (A) containing RNA was enriched for interferon mRNA by centrifugation on a linear 5-20% (w/v) sucrose gradient. The RNA samples were heated to 80°C for 2 minutes, rapidly cooled, layered
30 over the gradient, and centrifuged for 20 hours at 30,000 rpm at 4°C in a Beckman SW-40 rotor. Fractions were collec-ted, ethanol precipitated, and dissolved in H_2O.

One microgram samples of mRNA were injected into
35 Xenopus laevis oocytes as described by Cavalieri et al., Proc. Natl. Acad. Sci. USA 74, 3287-3291 [1977]. The injected oocytes were incubated 24 hours at 21°C, homo-genized, and centrifuged for 5 minutes at 10,000 x g. The

interferon in the supernatant was determined by the cyto-
pathic effect (CPE) inhibition assay (Stewart, The Inter-
feron System, Springer-Verlag, New-York-Wien, 1979) using
Sindbis virus and human diploid cells (WISH). Interferon
5   titers of 1,000 to 6,000 units recovered (NIH reference
standard) per microgram of RNA injected were routinely
obtained for the 12S species of mRNA.

F.   Synthesis and cloning of cDNA

10

      Single stranded cDNA was prepared in 100 µl reactions
containing 5 µg of 12S fraction mRNA, 20 mM Tris-HCl (pH
8.3), 20 mM KCl, 8mM $MgCl_2$, 30 mM β-mercaptoethanol, 100 µCi
of (α^{32}P)dCTP and 1 mM dATP, dCTP, dGTP, dTTP. The primer
15 was the synthetic HindIII decamer dCCAAGCTTGG (Scheller
et al., Science 196, 177-180 [1977]), which had been
extended at the 3'-terminus with about 20 to 30 deoxy-
thymidine residues using terminal deoxynucleotidyl trans-
ferase (Chang et al., Nature 275, 617-624 [1978]). 100 units
20 of reverse transcriptase were added and the reaction mix-
ture was incubated at 42°C for 30 minutes. The second
strand DNA synthesis was carried out as described previ-
ously (Goeddel et al., Nature 281, 544-548 [1979]). The
double stranded cDNA was treated with 1200 units of S1
25 nuclease for 2 hours at 37°C in 25 mM sodium acetate (pH
4.5), 1mM $ZnCl_2$, 0.3M NaCl. After phenol extraction the
mixture was separated electrophoretically on a 8% poly-
acrylamide gel. cDNA (∼ 0.5 µg) ranging from 550 to 1500
base pairs in size was recovered by electroelution. A 20 ng
30 aliquot was extended with deoxyC residues using terminal
deoxynucleotidyl transferase (Chang et al., supra), and
annealed with 100 ng of pBR322 which had been cleaved with
PstI and tailed with deoxyG residues (Chang et al., supra).
The annealed mixture was used to transform E. coli K-12
35 strain 294 by a published procedure (Hershfield et al.,
Proc. Natl. Acad. Sci. USA 71, 3455-3459 [1974]).

G.   Preparation of induced and uninduced ^{32}P-cDNA probes

5 µg of 12S mRNA were combined with either 2 µg of
oligo (dT)$_{12-18}$ or 5 µg of each synthetic primer pool
5 (Figure 1) in 60 µl of 10mM Tris-HCl (pH 8), 1 mM EDTA.
The mixtures were boiled 3 minutes, and quenched on ice.
60 µl of 40 mM Tris-HCl (pH 8.3), 40 mM KCl, 16mM MgCl$_2$,
60 mM β-mercaptoethanol, 1 mM dATP, dGTP, dTTP and 5 x
10^{-7} M (α-^{32}P) dCTP (2,000 - 3,000 Ci/mM) was added to each
10 template-primer mixture at 0°C. After the addition of
100 units of reverse transcriptase, the reactions were
incubated at 42°C for 30 minutes and purified by passage
over 10 ml Sephadex G-50 columns. The products were treated
with 0.3N NaOH for 30 minutes at 70°C, neutralized, and
15 ethanol precipitated.

The ^{32}P-cDNAs were combined with 100 µg of poly(A)
mRNA from uninduced fibroblasts in 50 µl of 0.4M sodium
phosphate (pH 6.8), 0.1% sodium dodecyl sulfate (SDS). The
20 mixtures were heated at 98°C for 5 minutes and allowed
to anneal 15 hours at 45°C. The DNA-RNA hybrids (con-
taining uninduced cDNA sequences) were separated from
single-stranded DNA (induced cDNA sequences) by chromato-
graphy on hydroxyapatite as described by Galau et al.
25 (Proc. Natl. Acad. Sci. USA 74, 1020-1023 [1977]). The
DNA-RNA hybrids were treated with alkali to remove RNA.

H.   Screening of recombinant plasmids with ^{32}P-cDNA probes

30   Approximately 1 µg samples of plasmid DNA were prepared
from individual transformants by a published procedure
(Birnboim et al., Nucleic Acids Res. 7, 1513-1523 [1979]).
The DNA samples were linearized by digestion with EcoRI,
denatured in alkali, and applied to each of three nitro-
35 cellulose filters by the dot hybridization procedure
(Kafatos et al., Nucleic Acids Res. 7, 1541-1552 [1979]).
The filters were hybridized with the ^{32}P-cDNA probes for
16 hours at 42°C in 50% formamide, 10x Denhardt's solution

(Biochem. Biophys. Res. Comm. 23, 641-646 [1966]), 6xSSC,
40 mM Tris-HCl (pH 7.5), 2mM EDTA, 40 µg/ml yeast RNA.
Filters were washed with 0.1xSSC, 0.1% SDS twice for
30 minutes at 42°C, dried, and exposed to Kodak XR-2 x-ray
5 film using Dupont Lightning-Plus intensifying screens at
-80°C. [SSC contains 0.15 M NaCl and 0.015 M sodium citrate,
pH 7.0].

I.   Construction of plasmids for direct expression of FIF

10

The synthetic primers I (dATGAGCTACAAC) and II
(dCATGAGCTACAAC) were phosphorylated using T4 polynucleo-
tide kinase and $(\gamma-^{32}P)$ATP to a specific activity of
700 Ci/mM as described by Goeddel et al., Proc. Natl. Acad.
15 Sci. USA 76, 106-110 [1979]. Primer repair reactions were
performed as follows: 250 pM of the ^{32}P-primers were
combined with 8 µg (10 pM) of a 1200 bp HhaI restriction
fragment containing the FIF cDNA sequence. The mixture
was ethanol precipitated, resuspended in 50 µl H_2O, boiled
20 3 minutes, quenched in a dry ice-ethanol bath, and com-
bined with a 50 µl solution of 20mM Tris-HCl (pH 7.5),
14 mM $MgCl_2$, 120 mM NaCl, 0.5 mM dATP, dCTP, dGTP, dTTP
at 0°C. 10 units of DNA polymerase I Klenow fragment were
added and the mixture was incubated at 37°C for 4 1/2
25 hours. Following extraction with phenol/$CHCl_3$ and restric-
tion with PstI, the desired product was purified on a 6%
polyacrylamide gel. Subsequent ligations were done at
room temperature (cohesive termini) or 4°C (blunt ends)
using conditions reported previously (Goeddel et al., supra).

30

J.   Assay for interferon expression in E. coli

Bacterial extracts were prepared for IF assay as
follows: One ml cultures were grown overnight in LB (Luria-
35 Bertani) medium containing 5 µg/ml tetracycline, then
diluted into 25 ml of M9 medium supplemented with 0.2%
glucose, 0.5% casamino acids and 5 µg/ml tetracycline.
10 ml samples were harvested by centrifugation when

absorbance at 550 nm (A_{550}) reached 1.0. The cell pellets
were quickly frozen in a dry ice-ethanol bath and cleared
lysates were prepared as described by Clewell (supra).
Interferon activity in the supernatants was determined by
5 comparison with NIH FIF standards using CPE inhibition
assays. Two different assays were used: (a) WISH (human
amnion) cells were seeded in microtiter dishes. Samples
were added 16 to 20 hours later and diluted by serial
2-fold dilution. Sindbis virus was added after at least
10 3 hours of incubation. Plates were stained 20 to 24 hours
later with crystal violet. (b) MDBK (bovine kidney) cell
line was seeded simultaneously with 2-fold dilutions of
samples. Vesicular stomatitis virus was added after 2 to 3
hours incubation and plates were stained with crystal
15 violet 16 to 18 hours later. To test pH 2 stability bac-
terial extracts and standards were diluted in minimal
essential medium to a concentration of 1000 units/ml. One
ml aliquots were adjusted to pH 2 with 1N HCl, incubated
at 4°C for 16 hours, and neutralized by addition of NaOH.
20 IF activity was determined by the CPE inhibition assay
using human amnion cells. To establish antigenic identity
25 µl aliquots of the 1000 U/ml interferon samples
(untreated) were incubated with 25 µl of rabbit antihuman
leukocyte interferon for 60 minutes at 37°C, centrifuged
25 at 12,000 x g for 5 minutes and the supernatant assayed.
Fibroblast and leukocyte interferon standards were obtained
from the National Institutes of Health. Rabbit antihuman
leukocyte interferon was obtained from the National Insti-
tute of Allergy and Infectious Diseases.
30

K,   Chemical synthesis of primer pools complementary to
      FIF mRNA

The known amino-terminal protein sequence of human
35 fibroblast interferon permitted us to deduce the 24 possible
mRNA sequences which could code for the first four amino
acids. The 24 complementary deoxyoligonucleotides were
synthesized in 6 pools of 4 dodecamers each (Figure 1).

The six pools of 4 deoxyoligonucleotides each were
synthesized by the modified phosphotriester method in
solution and on solid phase (Crea et al., supra). The
basic strategy involved reacting two different 3'-blocked
5　trimers with an excess of a single 5'-protected trimer to
yield a pool of two hexamers, each represented equally.
The coupling of two pools, each containing two hexamers,
then resulted in a pool of four dodecamers.

10　L.　　Identification of FIF cDNA clones

Using 12S mRNA from induced human fibroblasts (1,000
units IF activity per µg in oocyte assay), double stranded
cDNA was prepared and inserted into pBR322 at the PstI
15　site by the standard dG:dC tailing method as described
by Chang et al., supra. A fibroblast cDNA library con-
sisting of 30,000 ampicillin-sensitive, tetracycline-
resistant transformants of E. coli K-12 strain 294 was
obtained from 20 ng of cDNA ranging in size from 550 to
20　1300 base pairs. Plasmid DNA was prepared from 600 of the
transformants and applied to 3 sets of nitrocellulose
filters as described above.

The approach followed in the identification of hybrid
25　plasmids containing fibroblast interferon cDNA sequences
was similar to that used to identify human leukocyte inter-
feron recombinant plasmids (Goeddel et al., Nature 287,
411-416 [1980]). Radiolabeled cDNA hybridization probes
were prepared using either the 24 synthetic dodecamers
30　or oligo(dT)$_{12-18}$ as primers and 12S RNA from induced
fibroblasts (5000 units/µg in oocytes) as template. The
^{32}P-cDNAs (specific activity $>$ 5 x 10^8 cpm/µg) obtained
were hybridized to a large excess of mRNA isolated from
uninduced human fibroblasts, and the mRNA-cDNA hybrids
35　were separated from unreacted cDNA by hydroxyapatite
chromatography (Galau et al., supra). The single stranded
cDNA fractions should be enriched for sequences which are
present in induced fibroblasts but absent in uninduced

cells, and the mRNA-cDNA hybrids should represent sequences
common to both induced and uninduced cells. Approximately
4×10^6 cpm of single stranded cDNA (hybridization probe A)
and 8×10^6 cpm of cDNA-mRNA hybrids were obtained using
5   oligo(dT)$_{12-18}$ primed cDNA; 1.5×10^6 cpm of single
stranded (hybridization probe B) and 1.5×10^6 cpm of
hybrids were obtained from cDNA primed using synthetic
dodecamer pools 1-6. The cDNA-mRNA hybrids from both frac-
tionations were combined, the RNA hydrolyzed by treatment
10 with alkali, and the ^{32}P-cDNA used as hybridization probe
C. Many of the 600 plasmid samples hybridized with both
probes A and C, indicating that the hybridization reac-
tions between uninduced mRNA and ^{32}P-cDNA (prior to the
hydroxyapatite fractionation step) had not gone to comple-
15 tion. However, only one of the 600 plasmids (pF526) hybrid-
ized strongly with the specifically primed, induced cDNA
probe B (Figure 2). Plasmid pF526 also hybridized with the
total oligo(dT)$_{12-18}$ primed, induced cDNA probe A, and
failed to give detectable hybridization to the combined
20 uninduced probe C.

PstI digestion of pF526 showed the cloned cDNA insert
to be about 550 base pairs long, probably too short to
contain the entire coding region for fibroblast inter-
25 feron. Therefore, a ^{32}P-labeled DNA probe was prepared
from this PstI fragment by random priming with calf thymus
DNA (Taylor et al., supra). This probe was used to screen
2000 individual colonies from a newly constructed fibro-
blast cDNA library (the new cDNA library was prepared
30 using 12S mRNA from induced fibroblasts having a titer of
6,000 units/ml in the oocyte assay system). Sixteen clones
hybridized to the probe. Plasmids prepared from the
majority of these released two fragments when cleaved
with PstI, indicating that the cDNA contained an internal
35 PstI site. Clone pFIF3 contained the largest cDNA inert,
about 800 base pairs. The DNA sequence of the insert was
determined by the Maxam-Gilbert procedure (supra) and is
shown in Figure 3. The amino acid sequence of human fibro-

blast interferon predicted from the nucleotide sequence
is identical to that reported recently by Taniguchi et al.
(Gene 10, 11-15 [1980]) and by Derynck et al. (supra)
from DNA sequencing of FIF cDNA clones. A precursor or
5 signal peptide of 21 amino acids is followed by a sequence
of 166 amino acids representing the mature interferon,
a stretch of 196 3'-untranslated nucleotides and a poly(A)
tail. The NH_2-terminal 20 amino acids of mature FIF have
been direclty determined by protein microsequencing and
10 are the same as those predicted from the DNA sequence.

M.   Direct expression of fibroblast interferon

       To express high levels of mature fibroblast inter-
15 feron in E. coli initiation of protein synthesis must
occur at the ATG codon of the mature polypeptide (amino
acid 1) rather than at the ATG of the signal peptide
(amino acid S1) (Figure 3).

20   Our approach to removing the signal peptide coding
regions from pFIF3 is depicted in Figure 4. A 1200 bp DNA
fragment which contained the entire cDNA insert was
isolated from a polyacrylamide gel after digesting pFIF3
with HhaI. Two separate synthetic deoxyoligonucleotide
25 primers, dATGAGCTACAAC(I) and dCATGAGCTACAAC(II), were
prepared. Both primers contain the coding sequence for
the first four amino acids of mature fibroblast inter-
feron; primer II has an additional C at the 5'-terminus.
Primer repair reactions and subsequent ligations were
30 carried out separately for primers I and II, and gave
nearly identical results. Therefore, only reactions using
primer I are discussed in detail here. The primers were
5'-radiolabeled using $(\gamma-^{32}P)$ATP and T4 polynucleotide
kinase, combined with the 1200 bp HhaI DNA fragment and
35 the mixtures were denatured by boiling. Following hybridi-
zation of the primer to the denatured HhaI DNA fragment,
E. coli DNA polymerase I Klenow fragment (Klenow et al.,

Proc. Natl. Acad. Sci. USA 65, 168-175 [1970]) was used
to catalyze the repair synthesis of the plus (top) strand
(Figure 4). In addition, the associated $3' \longrightarrow 5'$ exo-
nuclease activity of the Klenow fragment removed the 3'-
5   protruding end from the minus (bottom) strand, leaving a
flush end. Analysis of samples of the reaction mixture
by polyacrylamide gel electrophoresis indicated that the
repair synthesis did not go to completion, but stopped
at several discrete sites. Therefore, the entire reaction
10 mixture was treated with PstI and the desired 141 bp frag-
ment (180,000 Cerenkow cpm; $\sim$0.3 pM) was purified by poly-
acrylamide gel electrophoresis (Figure 5). Ligation of this
fragment to 1 μg ($\sim$4 pM) of the 363 bp PstI-BglII frag-
ment isolated from pFIF3 (Fig. 4), followed by BglII
15 digestion, yielded 50,000 Cerenkov cpm ($\sim$0.1 pM, $\sim$30 ng)
of the 504 bp DNA fragment containing the entire coding
sequence for mature fibroblast interferon. The same reac-
tions using primer II gave 83,000 cpm ($\sim$0.15 pM, $\sim$50 ng)
of 505 bp product.
20

The construction of plasmids which direct the syn-
thesis of human fibroblast interferon is outlined in
Figure 6. Separate expression plasmids were constructed
which placed FIF synthesis under the control of the E. coli
25 lac or trp promoter-operator systems. Both ot these
systems have proven useful for the direct expression of
eukaryotic genes in E. coli: human growth hormone has been
efficiently synthesized using the lac system (Goeddel et
al., Nature 281, 544-548 [1979]) and human leukocyte inter-
30 feron has been produced at high levels using the trp
system (Goeddel et al., Nature 287, 411 [1980]).

pBRH trp was digested with EcoRI restriction enzyme
and the resulting fragment isolated by PAGE and electro-
35 elution. EcoRI-digested plasmid pSom 11 (Itakura et al.,
Science 198, 1056-1063 [1977]); G.B. patent publication
no. 2 007 676 A) was combined with the above fragment.
The mixture was ligated with T_4 DNA ligase and the resul-

ting DNA transformed into E. coli K-12 strain 294 as previously described. Transformant bacteria were selected on amicillin-containing plates. Resulting ampicillin-resistant colonies were screened by colony hybridization

5　(Grunstein et al., supra) using as a probe the trp promoter-operator containing the above fragment isolated from pBRHtrp, which had been radioactively labelled with P^{32}. Several colonies shown positive by colony hybridization were selected, plasmid DNA was isolated and the orienta-

10　tion of the inserted fragments determined by restriction analysis employing restriction enzymes BglII and BamHI in double digestion. E. coli 294 containing the plasmid designated pSOM7Δ2, which has the trp promoter-operator fragment in the desired orientation was grown in LB medium

15　containing 10 µg/ml ampicillin. The cells were grown to optical density 1 (at 550 nM), collected by centrifugation and resuspended in M9 media in tenfold dilution. Cells were grown for 2-3 hours, again to optical density 1, then lysed and total cellular protein analyzed by SDS urea

20　(15%) PAGE (Maizel et al., Methods Virol. $\underline{5}$, 180-246 [1971]).

Plasmid pBR322 was HindIII digested and the protruding HindIII ends in turn digested with S1 nuclease.

25　The S1 nuclease digestion involved treatment of 10 µg of HindIII-cleaved pBR322 in 30 µl S1 buffer (0.3 M NaCl, 1 mM $ZnCl_2$, 25 mM sodium acetate, pH 4.5) with 300 units S1 nuclease for 30 minutes at 15°C. The reaction was stopped by the addition of 1 µl of 30 x S1 nuclease stop

30　solution (0.8M Tris base, 50 mM EDTA). The mixture was phenol extracted, chloroform extracted and ethanol preci-pitated, then EcoRI digested as previously described and the large fragment (1) obtained by PAGE procedure follo-wed by electroelution. The fragment obtained has a first

35　EcoRI sticky end and a second, blunt end whose coding strand begins with the nucleotide thymidine.

Plasmid pSom7Δ2, as prepared above, was BglII digested
and the BglII sticky ends resulting made double stranded
with the Klenow polymerase I procedure using all four
deoxynucleotide triphosphates. EcoRI cleavage of the
5 resulting product followed by PAGE and electroelution
of the small fragment (2) yielded a linear piece of DNA
containing the tryptophan promoter-operator and codons
of the LE' "proximal" sequence upstream from the BglII
site ("LE'(p)"). The product had an EcoRI end and a blunt
10 end resulting from filling in the BglII site. However,
the BglII site is reconstituted by ligation of the blunt
end of fragment (2) to the blunt end of fragment (1).
Thus, the two fragments were ligated in the presence of
T_4 DNA ligase to form the recirculated plasmid pHKY 10
15 which was propagated by transformation into competent E.
coli strain 294 cells.

Plasmid pGM1 carries the E. coli tryptophan operon
containing the deletion ΔLE1413 (Miozzari et al., J.
20 Bacteriology 133, 1457-1466 [1978]) and hence expresses
a fusion protein comprising the first 6 amino acids of the
trp leader and approximately the last third of the trp
E polypeptide (hereinafter referred to in conjunction as
LE'), as well as the trp D polypeptide in its entirety,
25 all under the control of the trp promoter-operator system.
The plasmid, 20 μg, was digested with the restriction
enzyme PvuII which cleaves the plasmid at five sites. The
gene fragments were next combined with EcoRI linkers
(consisting of a self complementary oligonucleotide of
30 the sequence: pCATGAATTCATG) providing an EcoRI cleavage
site for a later cloning into a plasmid containing an
EcoRI site. The 20 μg of DNA fragments obtained from
pGM1 were treated with 10 units T_4 DNA ligase in the
presence of 200 pico moles of the 5'-phosphorylated syn-
35 thetic oligonucleotide pCATGAATTCATG and in 20 μl T_4 DNA
ligase buffer (20mM Tris, pH 7.6, 0.5 mM ATP, 10 mM $MgCl_2$,
5 mM dithiothreitol) at 4°C overnight. The solution was
then heated 10 minutes at 70°C to halt ligation. The linkers

were cleaved by EcoRI digestion and the fragments, now
with EcoRI ends were separated using 5% PAGE and the three
largest fragments isolated from the gel by first staining
with ethidium bromide, locating the fragments with ultra-
5 violet light, and cutting from the gel the portions of
interest. Each gel fragment, with 300 microliters 0.1xTBE,
was placed in a dialysis bag and subjected to electro-
phoresis at 100 V for one hour in 0.1xTBE buffer (TBE
buffer contains: 10.8 gm Tris base, 5.5 gm boric acid,
10 0.09 gm Na_2EDTA in 1 liter H_2O). The aqueous solution
was collected from the dialysis bag, phenol extracted,
chloroform extracted and made 0.2 M sodium chloride, and
the DNA recovered in water after ethanol precipitation.
The trp promoter-operator containing gene with EcoRI sticky
15 ends was identified in the procedure next described, which
entails the insertion of fragments into a tetracycline
sensitive plasmid which, upon promoter-operator insertion,
becomes tetracycline resistant.

20   Plasmid pBRH1 (Rodriguez et al., Nucleic Acids
Research 6, 3267-3287 [1979]) expressed ampicillin resis-
tance and contains the gene for tetracycline resistance
but, there being no associated promoter, does not express
that resistance. The plasmid is accordingly tetracycline
25 sensitive. By introducing a promoter-operator system in
the EcoRI site, the plasmid can be made tetracycline
resistant.

      pBRH1 was digested with EcoRI and the enzyme removed
30 by phenol extraction followed by chloroform extraction
and recovered in water after ethanol precipitation. The
resulting DNA molecule was, in separate reaction mixtures,
combined with each of the three DNA fragments obtained
above and ligated with T_4 DNA ligase as previously descri-
35 bed. The DNA present in the reaction mixture was used to
transform competent E. coli K-12 strain 294 by standard
techniques (Hershfield et al., supra) and the bacteria

plated on LB plates containing 20 µg/ml ampicillin and
5 µg/ml tetracycline. Several tetracycline-resistant
colonies were selected, plasmid DNA isolated and the
presence of the desired fragment confirmed by restriction
5 enzyme analysis. The resulting plasmid is designated
pBRHtrp.

An EcoRI and BamHI digestion product of the viral
genome of hepatitis B was obtained by conventional means
10 and cloned into the EcoRI and BamHI sites of plasmid pGH6
(Goeddel et al., Nature 281, 544-548 [1979]) to form the
plasmid pHS32. Plasmid pHS32 was cleaved with XbaI,
phenol extracted, chloroform extracted and ethanol preci-
pitated. It was then treated with 1 µl E. coli polymerase
15 I, Klenow fragment, in 30 µl polymerase buffer (50 mM
potassium phosphate pH 7.4, 7mM $MgCl_2$, 1 mM β-mercapto-
ethanol) containing 0.1mM dTTP and 0.1mM dCTP for 30
minutes at 0°C then 2 hours at 37°C. This treatment causes
2 of the 4 nucleotides complementary to the 5' protruding
20 end of the XbaI cleavage site to be filled in:

$$5' \quad \text{CTAGA——} \qquad \longrightarrow \qquad 5' \quad \text{CTAGA——}$$
$$3' \qquad \text{T——} \qquad\qquad\qquad 3' \qquad \text{TCT——}$$
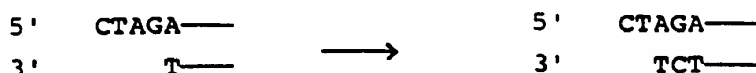
25   Two nucleotides, dC and dT, were incorporated giving
an end with two 5' protruding nucleotides. This linear
residue of plasmid pHS32 (after phenol and chloroform
extraction and recovery in water after ethanol precipi-
tation) was cleaved with EcoRI. The large plasmid frag-
30 ment was separated from the smaller EcoRI-XbaI fragment
by PAGE and isolated after electroelution. This DNA frag-
ment from pHS32 (0.2 µg) was ligated under conditions
similar to those described above, to the EcoRI-Taq I
fragment of the tryptophan operon (~0.01 µg), derived from
35 pBRHtrp.

In the process of ligating the fragment from pHS32
to the EcoRI-TaqI fragment, as described above, the

TaqI protruding end is ligated to the XbaI remaining
protruding end even though it is not completely Watson-
Crick base-paired:

5
```
        —T          CTAGA—                —TCTAGA—
             +                     ⟶
        —AGC         TCT—                —AGCTCT—
```
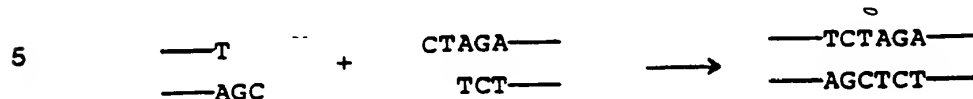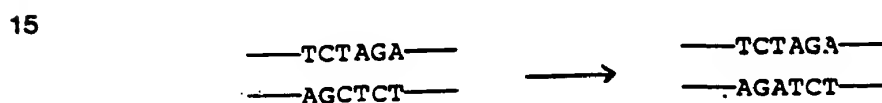
A portion of this ligation reaction mixture was
transformed into E. coli 294 cells, heat treated and
10 plated on LB plates containing ampicillin. Twenty-four
colonies were selected, grown in 3 ml LB media, and plas-
mid isolated. Six of these were found to have the XbaI
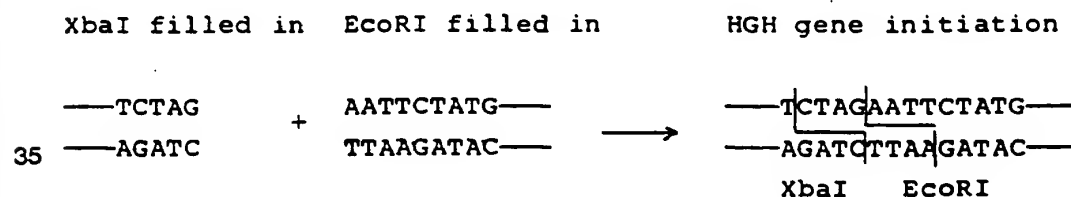site regenerated via E. coli catalyzed DNA repair and
replication.

15
```
        —TCTAGA—                —TCTAGA—
                        ⟶
        —AGCTCT—                —AGATCT—
```

These plasmids were also found to cleave both with
20 EcoRI and HpaI and to give the expected restriction frag-
ments. One plasmid, designated pTrp14, was used for
expression of heterologous polypeptides, as next discussed.

The plasmid pHGH 107 (Goeddel et al., Nature 281,
25 544-548 [1979]) contains a gene for human growth hormone
made up of 23 amino acid codons produced from synthetic
DNA fragments and 163 amino acid codons obtained from
complementary DNA produced via reverse transcription of
human growth hormone messenger RNA. This gene, though it
30 lacks the codons of the "pre" sequence of human growth
hormone, does contain an ATG translation initiation codon.
The gene was isolated from 10 µg pHGH 107 after treatment
with EcoRI followed by E. coli polymerase I Klenow frag-
ment and dTTP and dATP as described above. Following
35 phenol and chloroform extraction and ethanol precipitation
the plasmid was treated with BamHI.

The human growth hormone (HGH) gene-containing frag-
ment was isolated by PAGE followed by electroelution.
The resulting DNA fragment also contains the first 350
nucleotides of the tetracycline resistance structural
5 gene, but lacks the tetracycline promoter-operator system
so that, when subsequently cloned into an expression
plasmid, plasmids containing the insert can be located
by the restoration of tetracycline resistance. Because
the EcoRI end of the fragment has been filled in by the
10 Klenow polymerase I procedure, the fragment has one blunt
and one sticky end, ensuring proper orientation when
later inserted into an expression plasmid.

The expression plasmid pTrp14 was next prepared to
15 receive the HGH gene-containing fragment prepared above.
Thus, pTrp14 was XbaI digested and the resulting sticky
ends filled in with the Klenow polymerase I procedure
employing dATP, dTTP, dGTP and dCTP. After phenol and
chloroform extraction and ethanol precipitation the resul-
20 ting DNA was treated with BamHI and the resulting large
plasmid fragment isolated by PAGE and electroelution.
The pTrp14-derived fragment had one blunt and one sticky
end, permitting recombination in proper orientation with
the HGH gene containing fragment previously described.

25

The HGH gene fragment and the pTRP14 ΔXba-BamHI frag-
ment were combined and ligated together under conditions
similar to those described above. The filled in XbaI and
EcoRI ends ligated together by blunt end ligation to
30 recreate both the XbaI and the EcoRI site:

XbaI filled in   EcoRI filled in   HGH gene initiation

```
——TCTAG         AATTCTATG——           ——TCTAGAATTCTATG——
            +                      →
35 ——AGATC         TTAAGATAC——           ——AGATCTTAAGATAC——
                                           XbaI    EcoRI
```

This construction also recreates the tetracycline
resistance gene. Since the plasmid pHGH 107 expresses
tetracycline resistance from a promoter lying upstream
from the HGH gene (the lac promoter), this construction,
5 designated pHGH 207, permits expression of the gene for
tetracycline resistance under the control of the trypto-
phan promoter-operator. Thus the ligation mixture was
transformed into E. coli 294 and colonies selected on LB
plates containing 5 µg/ml tetracycline.

10

Plasmid pHGH 207 was EcoRI digested and the trp
promoter containing EcoRI fragment recovered by PAGE follo-
wed by electroelution. Plasmid pBRH1 was EcoRI digested
and the cleaved ends treated with bacterial alkaline phos-
15 phatase (BAP, 1 µg, in 50 mM Tris, pH 8, and 10 mM $MgCl_2$
for 30 min. at 65°C) to remove the phosphate groups on
the protruding EcoRI ends. Excess bacterial alkaline
phosphatase was removed by phenol extraction, chloroform
extraction and ethanol precipitation. The resulting linear
20 DNA, because it lacks phosphates on the protruding ends
thereof, will in ligation accept only inserts whose
complementary sticky ends are phosphorylated but will not
itself recircularize, permitting more facile screening
for plasmids containing the inserts.

25

The EcoRI fragment derived from pHGH 207 and the
linear DNA obtained from pBRH1 were combined in the
presence of T_4 ligase as previously described and ligated.
A portion of the resulting mixture was transformed into
30 E. coli strain 294 as previously described, plated on LB
media containing 5 µg/ml of tetracycline, and 12 tetra-
cycline resistant colonies selected. Plasmid was isolated
from each colony and examined for the presence of a DNA
insert by restriction endonuclease analysis employing
35 EcoRI and XbaI. One plasmid containing the insert was
designated pHKY1.

The plasmid pHKY10, described above, is a derivative
of pBR322 which contains a BglII site between the tetra-
cycline resistance (Tc^R) promoter and structural gene.
The large DNA fragment isolated after digesting pHKY10 with
5 PstI and BglII therefore contains part of the ampicillin
resistance (Ap^R) gene and all of the Tc^R structural gene,
but lacks the Tc^R promoter (Fig. 6). The plasmid pGH6
(Goeddel et al., Nature 281, 544-548 [1979]) was digested
with EcoRI, the resulting single stranded ends were filled
10 in with DNA polymerase I, and the plasmid was cleaved with
PstI. The small fragment, containing part of the Ap^R gene,
a double lac promoter and lac ribosome binding site, but
lacking an ATG initiation triplet was isolated. A similar
trp promoter fragment, containing the trp leader ribosome
15 binding site, but lacking an ATG sequence (Goeddel et al.,
Nature 287, 411-416 [1980]), may be isolated from pHKY1 described above.

The trp fragment just referred to is an analog of the
E. coli tryptophan operon from which the so-called trp
20 attenuator has been deleted (Miozzari et al., J. Bact.
133, 1457-1466 [1978]) to controllably heighten expression
levels. Expression plasmids containing the modified trp
regulon can be grown to predetermined levels in nutrient
media containing additive tryptophan in quantities suffi-
25 cient to repress the promoter-operator system, then be
deprived of tryptophan so as to derepress the system and
occasion the expression of the intended product.

The expression plasmids may be assembled via three
30 part ligation reactions as shown in Figure 6. 15 ng
(0.05 pM) of the assembled FIF gene (504 or 505 bp),
0.5 µg (0.2 pM) of the large PstI - BglII fragment of
pHKY10 and 0.2 µg (0.3 pM) of the appropriate promoter
fragment were ligated and the mixture used to transform
35 E. coli 294 (Goeddel et al., Nature 287, 411-416 [1980]).
Plasmid DNA was prepared from individual transformants
and analyzed by restriction mapping. Correct joining of
the assembled gene to the promoter fragment should restore

the EcoRI (lac) or the XbaI (trp) recognition sequences.
The majority of the plasmids gave the expected restriction
enzyme digestion patterns. Individual clones (12 containing
the trp promoter and 12 containing the lac promoter) were
5 grown and extracts prepared for interferon assay as des-
cribed above.

When assayed on human amnion (WISH) cells for anti-
viral activity by the CPE inhibition assay five of the trp
10 transformants were positive (each approximately equiva-
lent); eleven of the lac transformants gave equivalent IF
activities. Therefore, one transformant from each series
(pFIFlac9 and pFIFtrp69) was selected for further study
(Table 1). DNA sequence analysis demonstrated that the
15 desired attachment of promoter to FIF structural gene had
occurred in both cases.

Table 1. Interferon activity in extracts of E. coli

E. coli K-12 strain 294 transformed by	Cell density (cells/ml)	IF Activity (units/l culture)	FIF molecules per cell
pBR322	3.5×10^8	-	-
pFIFlac9	3.5×10^8	9.0×10^6	2,250
pFIFtrp69	3.5×10^8	1.8×10^7	4,500
pFIFtrp^{3}69	3.5×10^8	8.1×10^7	20,200

30 Cells were grown and extracts prepared as described
above. The human amnion (WISH) cell line was used for the
CPE inhibition assay. Activities given are the average
from three independent experiments. To determine the
number of IF molecules per cell a FIF specific activity
35 of 4×10^8 units/mg was used (Knight, supra).

The amounts of fibroblast interferon produced by
pFIFlac9 and pFIFtrp69 are shown in Table 1. The trp

promoter gave a FIF expression level measurable higher
than did the lac promoter. In an attempt to further
increase FIF expression levels, pFIFtrp69 was cleaved with
EcoRI and two 300 base pair EcoRI fragments containing
5   the trp promoter (Goeddel et al., Nature $\underline{287}$, 411-416
[1980]) were inserted. The resulting plasmid, pFIFtrp^{3}69,
contains three successive trp promoters which read toward
the FIF gene. The amount of FIF synthesized by E. coli
K-12 strain 294/pFIF trp^{3}69 is 4-5 times that produced by
10 pFIFtrp69 (Table 1). This is apparently due to the
derepression of the trp promoter which occurs when trp
repressor levels are titrated by the multiple copies of
the trp operator.

15   The FIF produced by E. coli K-12 strain 294/pFIFtrp69
behaves like authentic human FIF. As shown in Table 2,
its antiviral activity is about 30 times greater on human
cells than on bovine cells. In addition, the bacterially
produced FIF is stable to treatment at pH 2 overnight and
20 is not neutralized by rabbit antihuman leukocyte inter-
feron antibodies (Table 3).

Table 2. Interferon activities measured on different
cell types

25

Cells	Interferon Activity (units/ml)		
	LeIF	FIF	E. coli K-12 strain 294/pFIFtrp69 extract
Human amnion	20,000	10,000	1280
Bovine kidney	13,000	400	40

35   LeIF and FIF were NIH standard solutions having 20,000
units/ml and 10,000 units/ml respectively. Assays were
performed as described above.

Table 3. Comparison of activities of extracts from
E. coli K-12 strain 294/pFIFtrp69 with
standard human leukocyte and fibroblast
interferons

	Interferon Activity (units/ml)		
	LeIF	FIF	E. coli K-12 strain 294/pFIFtrp69
untreated	1000	1000	1000
pH 2	1000	1000	1000
rabbit antihuman LeIF antibodies	<16	1000	1000

Experimental procedures described above. Assayed by
CPE inhibition using WISH cells/Sindbis virus.

N. Purification

The purification procedure for bacterial derived fibro-
blast interferon is as follows:

1. Frozen cells are suspended in twelve times volume per
weight with sucrose lysis buffer (100mM Tris-HCl, 10%
sucrose, 0.2M NaCl, 50mM EDTA, 0.2mM PMSF [phenylmethyl-
sulfonyl chloride], pH 7.9) containing lysozyme at 1mg/ml.
The cell suspension is stirred for 1 hour at 4°C and
centrifuged. Fibroblast interferon activity remains in the
supernatant.

2. Polyethyleneimine (5%, v/v) is added to the sonicated
supernatant to a final concentration of 0.5% (v/v). The
solution is stirred for 1 hour at 4°C and centrifuged.
Interferon activity remains in the supernatant.

3.   Solid ammonium sulfate is added to the polyethylene-
imine supernatant to a final concentration of 50% satura-
tion, stirred for 30 minutes at 4°C and centrifuged. Inter-
feron activity is in the 50% pellet.

5

4.   The 50% ammonium sulfate pellet is suspended in one
half the volume of the 50% ammonium sulfate suspension
with PBS (20 mM sodium phosphate, 0.15M NaCl, pH 7.4).
Polyethylene glycol 6000 (50%, w/v, in PBS) is added to a
10 final concentration of 12.5% (v/v), stirred at 4°C for
2 hours and centrifuged. Interferon activity is in the
pellet. The pellet is suspended in a minimal volume of
sucrose lysis buffer and clarified by centrifugation.

15   This initial extraction procedure results in a puri-
fication of fibroblast interferon from 0.001% of the
total protein to 0.05% of the total protein. This material
can be further purified to homogeneity by the following
column chromatography steps:

20

5.   Afinity chromatography on Amicon Blue B in sucrose
lysis buffer.

6.   Anion exchange chromatography on QAE Sephadex in
25 sucrose lysis buffer in the absence of 0.2M NaCl.

7.   Size exclusion chromatography on Sephadex G-75 in
sucrose lysis buffer.

30 8.   Reverse phase high pressure liquid chromatography.

O.   <u>Parenteral Administration</u>

FIF may be parenterally administered to subjects
35 requiring antitumor or antiviral treatment. Dosage and
dose rate may parallel that currently in use in clinical
investigations of human derived materials, e.g., about
$(1-10) \times 10^6$ units daily, and in the case of materials of

purity greater than 1%, likely up to, e.g., 15×10^7 units
daily. Dosages of bacterially obtained FIF could be signi-
ficantly elevated for greater effect owing to the essen-
tial absence of human proteins other than FIF, which pro-
5   teins in fibroblast-derived materials may act as pyrogens,
exhibiting adverse effects, e.g., malaise, temperature
elevation, etc.

As one example of an appropriate dosage form for
10   essentially homogeneous bacterial FIF in parenteral form,
3 mg FIF of specific activity of, say, 2×10^8 U/mg may
be dissolved in 25 ml of 5% human serum albumin, the
solution is passed through a bacteriological filter and
the filtered solution aseptically subdividied into 100
15   vials, each containing 6×10^6 units pure interferon
suitable for parenteral administration. The vials are
preferably stored in the cold (-20°C) prior to use.

The compounds of the present invention can be formula-
20   ted according to known methods to prepare pharmaceuti-
cally useful compositions, whereby the polypeptide hereof
is combined in admixture with a pharmaceutically acceptable
carrier vehicle. Suitable vehicles and their formulation
are described in Remington's Pharmaceutical Sciences by
25   E.W. Martin, which is hereby incorporated by reference.
Such compositions will contain an effective amount of the
interferon protein hereof together with a suitable amount
of vehicle in order to prepare pharmaceutically acceptable
compositions suitable for effective administration to the
30   host. One preferred mode of administration is parenteral.

35

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What we claim is:

1. A polypeptide comprising the amino acid sequence of a mature human fibroblast interferon, microbially pro-
5   duced and unaccompanied by any corresponding presequence or portion thereof.

2. A polypeptide according to claim 1, unaccompanied by associated glycosylation.
10

3. The polypeptide according to claim 1, optionally containing the amino acid methionine as the ordinarily first amino acid of said interferon.

15   4. The polypeptide according to claim 1, optionally containing a cleavable conjugate or microbial signal pro-tein attached to the N-terminus of the ordinarily first amino acid of said interferon.

20   5. A DNA sequence comprising a sequence coding for the polypeptide according to claims 1, 3 or 4.

6. The DNA sequence according to claim 5 operably linked with a DNA sequence capable of effecting microbial
25   expression of a polypeptide according to claims 1, 3 or 4.

7. A replicable microbial expression vehicle capable, in a transformant microorganism, of expressing a poly-peptide according to claims 1, 3 or 4.
30

8. A microbial expression vehicle according to claim 7 which is a plasmid.

9. A plasmid selected from the group consisting of
35 pFIFlac9, pFIFtrp69, and pFIFtrp^{3}69.

10. A microorganism transformed with an expression vehicle according to any one of claims 7-9.

11. The microorganism according to claim 10, obtained by transforming an E. coli strain.

12. A transformed microorganism according to claim 11 wherein said E. coli strain is E. coli K-12 strain 294.

13. A transformed microorganism according to claim 10 obtained by transforming Bacillus subtilis.

14. A transformed microorganism according to claim 10 obtained by transforming Saccharomyces cerevisiae.

15. A composition of matter comprising a therapeutically active fraction of a polypeptide consisting essentially of the amino acid sequence of a mature human fibroblast interferon, the balance of said composition comprising soluble microbial protein from which said polypeptide may be purified to a degree sufficient for effective therapeutic application.

16. A bacterial extract comprising greater than about 95% pure polypeptide consisting essentially of the amino acid sequence of a mature fibroblast interferon according to any one of claims 1-4.

17. A pharmaceutical composition comprising a therapeutically effective amount of a mature human fibroblast interferon according to claim 3 or 4, suitable for pharmaceutical administration.

18. The composition according to claim 17 suitable for parenteral administration.

19. A culture of microbial cells capable of producing a human fibroblast interferon in mature form.

20. The use of a mature human fibroblast interferon
according to claims 1, 3 or 4, for antitumor or antiviral
treatment or for preparing pharmaceutical compositions
useful for such treatment.

5

21. A process for producing a polypeptide claimed in
any one of claims 1-4 which process comprises causing a
microorganism, transformed with a replicable microbial
expression vehicle capable of expressing said polypeptide,
10 to grow up and express said polypeptide and recovering it.

22. A process for producing microorganisms capable
of expressing a polypeptide claimed in any one of claims
1-4 which process comprises transforming a microorganism
15 with a replicable microbial expression vehicle capable of
expressing said polypeptide and cultivating the trans-
formed microorganism.

23. A process for producing a replicable microbial
20 expression vehicle capable in a transformant microorganism
of expressing a polypeptide as claimed in any one of
claims 1-4, which process comprises constructing a first
DNA sequence coding for said polypeptide and operably
linking said first DNA sequence with a second DNA sequence
25 capable of effecting microbial expression of said first
DNA sequence.

24. The process of claim 23 wherein said second DNA
sequence comprises a multiple trp promoter-operator.

30

25. The products and processes for their preparation
as hereinbefore described.

***

35

What we claim is: A U S T R I A

1. A process for producing a polypeptide comprising
the amino acid sequence of a mature human fibroblast
5 interferon unaccompanied by any corresponding presequence
or portion thereof, which process comprises causing a micro-
organism transformed with a replicable microbial ex-
pression vehicle capable of expressing said polypeptide,
to grow up and to express said polypeptide and recovering
10 it.

2. A process as claimed in claim 1, wherein the
polypeptide is unaccompanied by associated glycosylation.

15 3. A process as claimed in claim 1, wherein the poly-
peptide optionally contains the amino acid methionine as
the ordinarily first amino acid of said fibroblast inter-
feron.

20 4. A process as claimed in claim 1, wherein the poly-
peptide optionally contains a cleavable conjugate or micro-
bial signal protein attached to the N-terminus of the
ordinarily first amino acid of said fibroblast interferon.

25 5. A process for the production of microorganisms
capable of producing a polypeptide comprising the amino
acid sequence of a mature human fibroblast interferon
unaccompanied by any corresponding presequence or portion
thereof, which process comprises transforming a micro-
30 organism with a replicable microbial expression vehicle
capable of expressing said polypeptide and cultivating
the transformed microorganism.

6. A process for the production of a replicable
35 microbial expression vehicle capable in a transformant
microorganism of expressing a polypeptide comprising the
amino acid sequence of a mature human fibroblast interferon
unaccompanied by any corresponding presequence or portion

thereof which process comprises constructing a first DNA
sequence coding for said polypeptide and operably linking
said first DNA sequence with a second DNA sequence capable
of effecting microbial expression of said first DNA
5   sequence.

    7. The process of claim 6 wherein said second DNA
sequence comprises a multiple trp promoter-operator.

10   8. A process for the preparation of pharmaceutical
compositions containing a microbially produced polypeptide
comprising the amino acid sequence of a mature human
fibroblast interferon unaccompanied by any corresponding
presequence or portion thereof which process comprises
15 mixing said polypeptide with non-toxic, inert, thera-
peutically compatible carriers and bringing the resulting
mixture into a suitable pharmaceutical dosage form.

    9. A pharmaceutical composition containing a micro-
20 bially produced polypeptide comprising the amino acid
sequence of a mature human fibroblast interferon unaccom-
panied by any corresponding presequence or portion thereof
and a non-toxic, inert, therapeutically compatible carrier
material.
25

    10. The processes for the preparation of polypeptides,
replicable microbial expression vehicles and microorganisms
as hereinbefore described.

30   11. A DNA sequence comprising a sequence coding for
the amino acid sequence of a mature human fibroblast
interferon unaccompanied by any corresponding presequence
or portion thereof.

35   12. A DNA sequence comprising a sequence coding for
the amino acid sequence of a mature human fibroblast
interferon the amino acid methionine optionally being the
ordinarily first amino acid of said interferon.

13. A DNA sequence comprising a sequence coding for
the amino acid sequence of a mature human fibroblast
interferon as well as for the amino acid sequence of a
cleavable conjugate or microbial signal protein attached
5 to the N-terminus of the ordinarily first amino acid
of said interferon.

14. A DNA sequence according to any one of claims
11-13 operably linked with a DNA sequence capable of
10 effecting microbial expression of the encoded polypeptide.

15. A replicable microbial expression vehicle capable,
in a transformant microorganism, of expressing a poly-
peptide coded by a DNA sequence according to any one of
15 claims 11-14.

16. An expression vehicle as claimed in claim 15
which is a plasmid.

20   17. A plasmid selected from the group consisting of
pFIFlac9, pFIFtrp69 and $pFIFtrp^{3}69$.

18. A microorganism transformed with an expression
vehicle according to any one of claims 15-17.
25
19. The microorganism according to claim 18 obtained
by transforming an E. coli strain.

20. A transformed microorganism according to claim
30 19 wherein said E. coli strain is E. coli K-12 strain 294.

21. A transformed microorganism according to claim
18 obtained by transforming Bacillus subtilis.

35   22. A transformed microorganism according to claim
18 obtained by transforming Saccharomyces cerevisiae.

Protein

$$\begin{array}{cccc} 1 & 2 & 3 & 4 \\ \text{Met} & \text{Ser} & \text{Tyr} & \text{Asn} \end{array}$$
Met–Ser–Tyr–Asn–

mRNA

$(5')$ AUG–UC$^{\mathrm{G}}_{\mathrm{A}}{}^{\mathrm{A}}_{\mathrm{U}}$-UA$^{\mathrm{U}}_{\mathrm{C}}$–AA$^{\mathrm{U}}_{\mathrm{C}}$ (16 combinations)
       ·C

$(5')$ AUG–AG$^{\mathrm{U}}_{\mathrm{C}}$–UA$^{\mathrm{U}}_{\mathrm{C}}$–AA$^{\mathrm{C}}_{\mathrm{U}}$ (8 combinations)

Complementary DNA primers

ATT–$^{\mathrm{A}}_{\mathrm{G}}$TA–$^{\mathrm{T}}_{\mathrm{C}}$GA–CAT   Pool 1

ATT–$^{\mathrm{A}}_{\mathrm{G}}$TA–$^{\mathrm{A}}_{\mathrm{G}}$GA–CAT   Pool 2

ATT–$^{\mathrm{A}}_{\mathrm{G}}$TA–$^{\mathrm{A}}_{\mathrm{G}}$CT–CAT   Pool 3

GTT–$^{\mathrm{A}}_{\mathrm{G}}$TA–$^{\mathrm{T}}_{\mathrm{C}}$GA–CAT   Pool 4

GTT–$^{\mathrm{A}}_{\mathrm{G}}$TA–$^{\mathrm{A}}_{\mathrm{G}}$GA–CAT   Pool 5

GTT–$^{\mathrm{A}}_{\mathrm{G}}$TA–$^{\mathrm{A}}_{\mathrm{G}}$CT–CAT   Pool 6

FIG. 1.

FIG. 2.

5'
S1   S10   S20 S21 MET SER TYR ASN
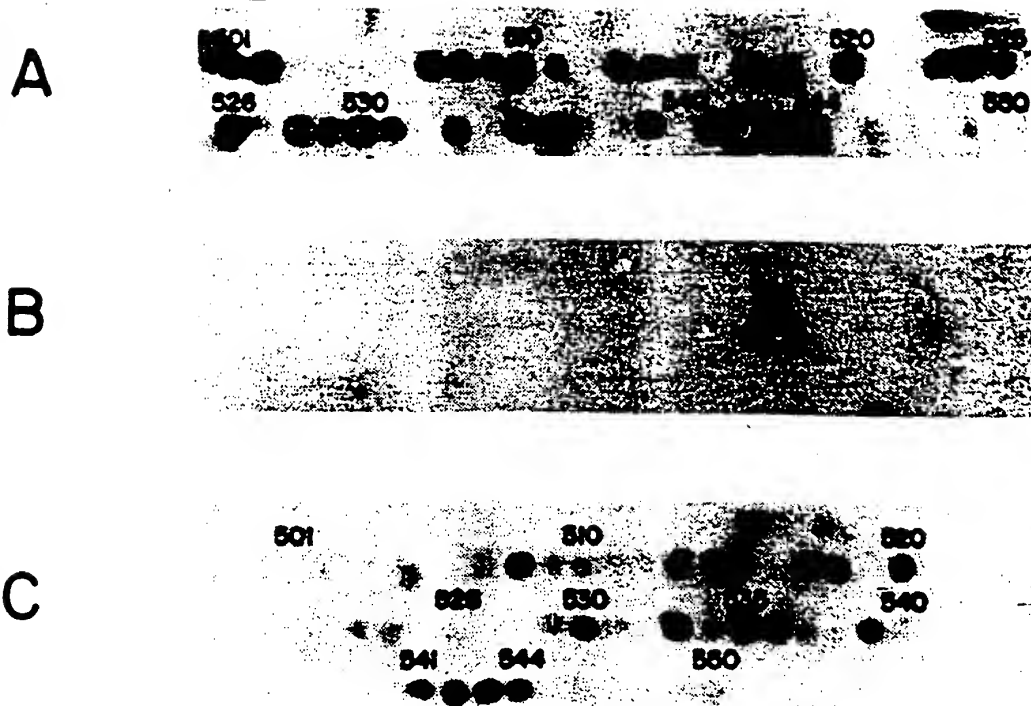met thr asn lys cys leu leu gln ile ala leu leu leu cys phe ser thr thr ala leu ser MET SER TYR ASN
ATG ACC AAC AAG TGT CTC CTC CAA ATT GCT CTC CTG TTG TGC TTC TCC ACT ACA GCT CTT TCC ATG AGC TAC AAC
                                                                    50

                        10   20
LEU LEU GLY PHE LEU GLN ARG SER SER ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU
TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA
                                100   150

30   40   50
TYR CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU GLN GLN PHE GLN LYS GLU ASP
TAT TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC
                                                        200

                        60   70
ALA ALA LEU THR ILE TYR GLU MET LEU GLN ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP
GCC GCA TTG ACC ATC TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG
                                250   300

80   90
ASN GLU THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU LYS THR VAL LEU GLU GLU
AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA
                                                350

                        110   120
LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE
AAA CTG GAG AAA GAA GAT TTT ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA AGA TAT TAT GGG AGG ATT
                                400   450

130   140   150
LEU HIS TYR LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG ASN PHE
CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT
                                                500

                160   166
TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN END
TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC TGA AGATCTCCTAGCCTGTCCCTCTGGGACTGGACAATTGCTTCAAGCA
                        550   600

TTCTTCAACCAGCAGATGCTGTTTAAGTGACTGATGGCTAATGTACTGCAAATGAAAGGACACTAGAAGATTTTGAAATTTTTATTAAATTATGAGTT
                                        650   700

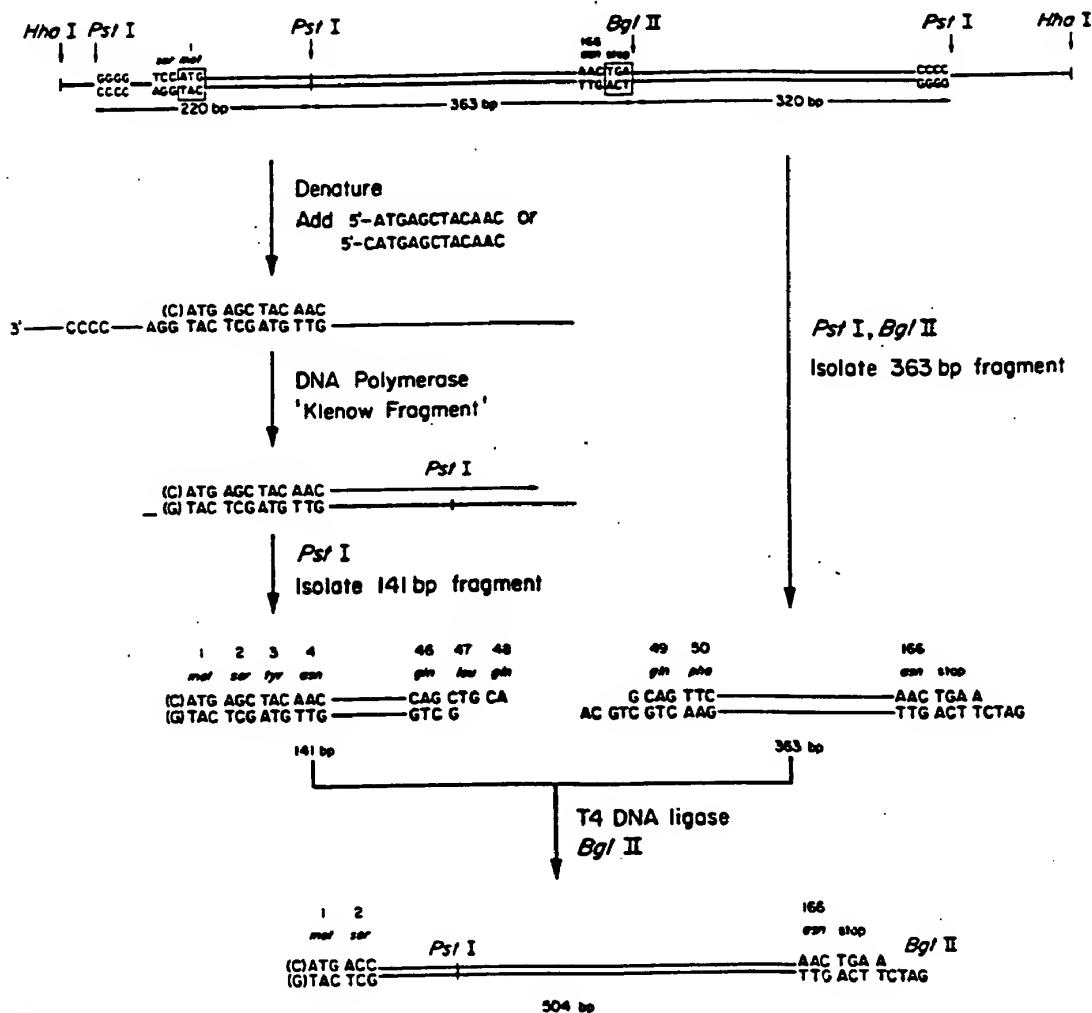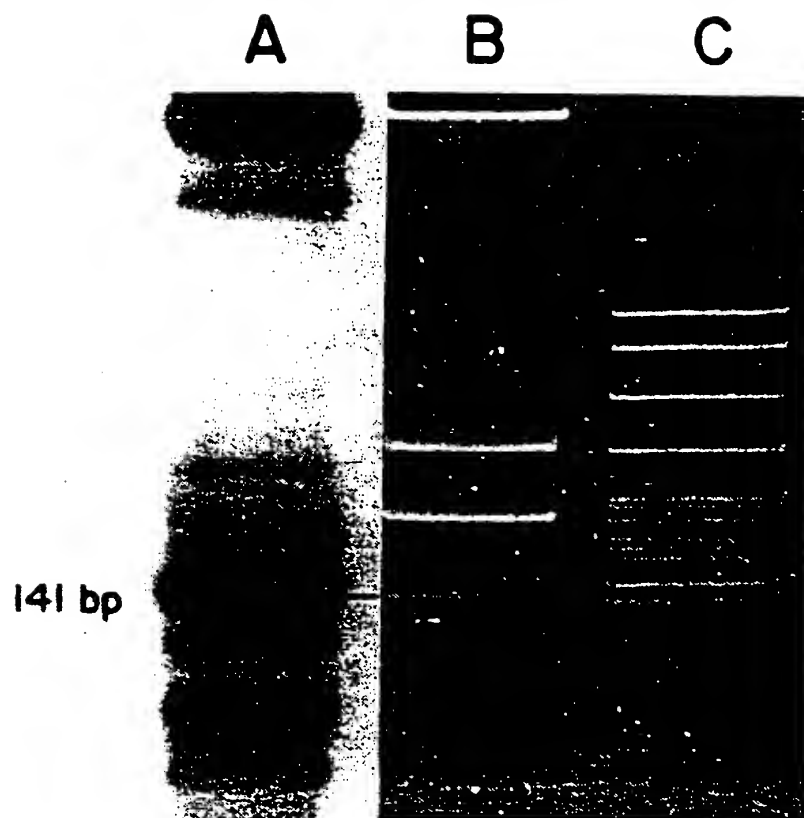ATTTTTATTTATTTAAATTTTATTTTGGAAAATAAATTATTTTTGGTGCAAAA
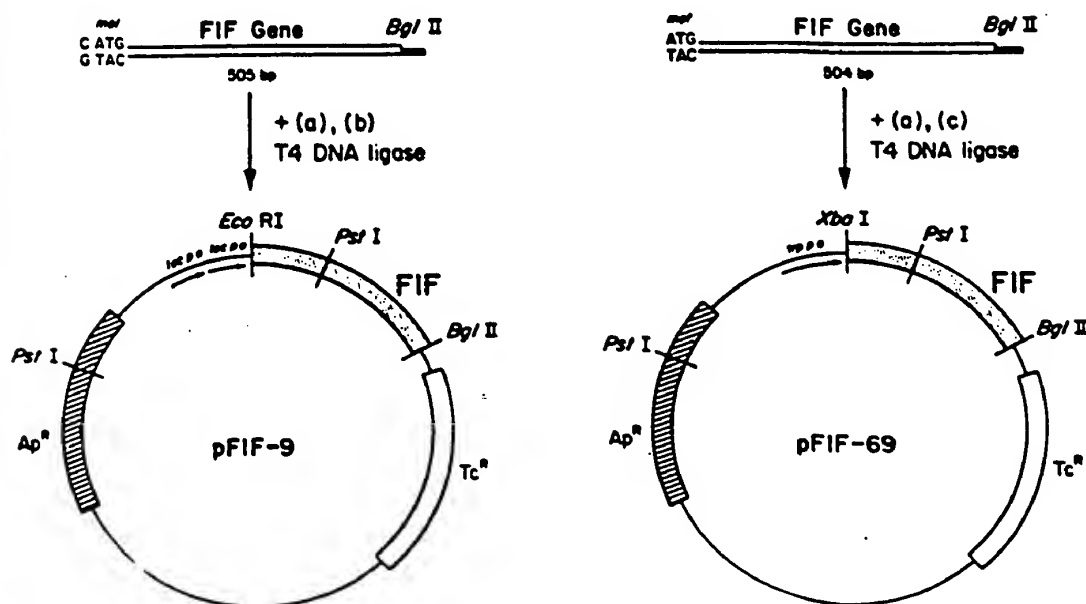   750
                                        3'

FIG. 3.

FIG. 4.

141 bp

FIG. 5.

FIG.6.